

A2 The present invention is related to a method for obtaining a surface activation of a solid support that allows the binding of molecules (capture nucleotide sequences, capture antibodies, receptors etc.) by a functionalization of said solid support surface in order to improve the building of microarrays.

Please replace the paragraph beginning at page 1, line 11, with the following rewritten paragraph:

A3 Description of the Related Art

Please replace the paragraph beginning at page 1, line 12, with the following rewritten paragraph:

A4 Microarrays are powerful tools for simultaneous detection of many different target molecules present in a sample, preferably biomolecules like nucleotide sequences, ligands, antibodies, etc. For DNA biochips, the binding properties of the molecules present upon the array depend mainly on the number, the sequence and the length of the capture nucleotide sequences and the way they are addressed onto the support. DNA biochip technology uses microscopic arrays of DNA molecules immobilized on solid supports. Biochip microarray applications are numerous and used for biomedical analysis such as gene expression analysis, polymorphism or mutation detection, molecular diagnostic, DNA sequencing and gene discovery (Ramsay et al., *Nature Biotechnology* 16, p. 40 (1998)).

Please replace the paragraph beginning at page 1, line 22, with the following rewritten paragraph:

A5 Such DNA microarrays are prepared by various methodologies. DNA can be synthesized *in situ* on glass surface by using combinatorial chemistry (Pease et al., *Proc. Natl. Acad. Sci. USA* 91, p. 5022 (1994)). This methodology produces DNA microarrays consisting of groups of oligonucleotides ranging in size from 10-25 bases whereas DNA microarrays prepared by micro-deposition with a robot can be of any length going from small oligonucleotides to 0.5-2 kb nucleotide sequences obtained for example after amplification by the polymerase chain reaction (PCR) (Zammatteo et al., *Anal. Biochem.* 253, p. 180 (1997)). Mechanical microspotting uses passive (pins) or active (ink jet nozzles) devices to deliver small quantities of DNA onto known regions.

Please replace the paragraph beginning at page 1, line 31, with the following rewritten paragraph:

A6
Glass is a popular substrate for DNA biochips, primarily due to its low fluorescence, transparency, low cost and resistance to high temperature and many chemical reagents (Cheung et al., *Nature Genetics supplement* 21, p. 15 (1999)). It has a number of practical advantages over porous membranes and gel pads. Liquid cannot penetrate the surface of the support and target nucleotide sequences have direct access to corresponding capture nucleotide sequences without diffusing into pores (Southern et al., *Nature Genetics supplements* 21, p. 5 (1999)). Microscope slides are currently used in laboratories because they are easy to handle and adaptable to automatic readers.

Please replace the paragraph beginning at page 2, line 7, with the following rewritten paragraph:

A7
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Modifications of the glass surface properties by addition of polylysine (Skena et al., *Science* 270, p. 467 (1995)) or by hydrophobic coatings (Allemand et al., *Biophys. J.* 73, p. 2064 (1997)) have been proposed in order to obtain a direct binding of DNA nucleotide sequences. However, in these cases, the DNA strands are susceptible to removal from the surface under high salt or high temperature conditions. Therefore, covalent binding methods are preferred. DNA can be cross-linked by ultraviolet-irradiation to form covalent bonds between the thymidine residues in the DNA sequence and positively charged amine groups added on the functionalized slides (Duggan et al., *Nature Genetics supplement* 21, p. 10 (1999)). However, the location and the number of attached points of the DNA molecules are not well defined, so that the length and the sequences available for hybridization can vary with the fixation conditions. An alternative method is to fix DNA molecules by one of its extremities. Thus carboxylated (Joos et al., *Anal. Biochem.* 247, p. 96 (1997)) or phosphorylated DNA (Rasmussen et al., *Anal. Biochem.* 198, p. 138 (1991)) can be coupled on aminated support as well as the reciprocal situation (Ghosh et al., *Nucleic Acids Res.* 15, p. 5353 (1987)). Others have bound amino-terminal oligonucleotides to isothiocyanate activated glass (Guo et al., *Nucleic Acids Res.* 22, p. 5456 (1999)), to aldehyde activated glass (Skena et al., *Proc. Natl. Acad. Sci. USA* 93, p. 10614 (1996)) or to surfaces modified with epoxide (Lamtire et al., *Nucleic Acids Res.* 22, p. 2121 (1994)). Thiol modified or disulfide modified oligonucleotides were also grafted onto aminosilane via heterobifunctional crosslinker (Chrissey et al., *Nucleic Acids Res.* 24, p. 3031 (1996)) or on 3-mercaptopropyl silane (Rogers et al., *Anal. Biochem.* 266, p. 23 (1999)). However, in these cases, the binding was not stable at high temperature. Recently, a more elaborate chemistry has been proposed for the

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construction of tethered molecules on the glass to which DNA can be attached (Beier et al.,
Nucleic Acids Res. 27, p. 1970 (1999)).

Please replace the paragraph beginning at page 3, line 3, with the following
rewritten paragraph:

18
Recently, Zammattéo et al. (*Analytical Biochemistry* 280, p. 143 (2000)) compared
several coupling strategies currently used to covalently graft DNA onto a glass surface. They
tested the carbodiimide mediated coupling of aminated, carboxylated and phosphorylated DNA
on carboxylic acid or amine modified glass supports. These methods were compared with the
binding of aminated DNA to aldehyde activated glass. They concluded that the fixation of
aminated DNA to aldehyde modified surface gives the best coupling procedure to build DNA
microarrays in term of coupling yield, rate of reaction in the absence of coupling agent. Besides
glass, polymers are becoming increasingly used for microarray and for the miniaturization of the
biological assays due to the development of the microfluidic technology and the "lab on a chip"
concept. In order to perform the assays, biological or ligand molecules have to be fixed on the
surface of the polymer and the requirement of a simple method of polymer activation would be
valuable.

19
Please delete the paragraph beginning at page 3, line 17.

Please replace the paragraph beginning at page 3, line 18, with the following
rewritten paragraph:

20
The present invention provides a new process for obtaining a surface activation
(functionalization or modification) of a solid support that is easy and rapid to perform in order to
introduce functions upon said solid support suitable for covalent binding of bio- or
chemomolecules and for the building of improved biochips or chemochips microarrays.

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Please replace the paragraph beginning at page 3, line 23, with the following
rewritten paragraph:

22
A preferred embodiment of the present invention is to provide by said method, improved
chip microarrays that will increase the detection sensitivity (increased coupling yield, rate of
reaction, etc.) of target molecules upon the microarrays of said chips allowing their identification
and/or quantification and/or recovery.

23
Please replace the paragraph beginning at page 3, line 29, with the following
rewritten paragraph:

A11
The present invention is related to a method for the activation (modification or functionalization) of a solid support surface by an oxidation of chemical groups present upon said surface, allowing the formation of aldehyde functions at the surface of said solid support, said aldehyde functions being suitable for covalent coupling (binding or linkage) with biological or chemical molecules.

Please replace the paragraph beginning at page 4, line 10, with the following rewritten paragraph:

A12
Therefore, the present invention is related to a method for making microarrays, comprising the steps of:

Please replace the paragraph beginning at page 4, line 12, with the following rewritten paragraph:

- A13
a) submitting the surface of a solid support to an oxidation of chemical groups present upon said surface in order to allow the formation of aldehyde functions upon the surface of said solid support, and

Please replace the paragraph beginning at page 4, line 15, with the following rewritten paragraph:

- A14
b) covalently coupling upon said aldehyde functions capture molecules designed for the detection, the identification, the quantification and/or the recovery of complementary target biological or chemical molecules of interest; said covalent binding resulting in (being made in order to obtain) an array comprising a density of at least 4, 10, 16, 20 or much more discrete regions per cm^2 of solid support surface, each of said discrete surface regions being bound (linked) with a species of capture molecules.

Please replace the paragraph beginning at page 4, line 22, with the following rewritten paragraph:

A15
The locations of the discrete regions (or spots) have a diameter comprised preferably between 10 and 500 μm and are separate by distances of similar order of magnitude, so that the array of the solid support comprises between 10 and 250000 discrete regions or spots upon a surface of 1 cm^2 , but preferably between 10 and 1000 spots upon a surface of 1 cm^2 .

Please replace the paragraph beginning at page 4, line 32, with the following rewritten paragraph:

A16
According to other embodiments of the present invention, the solid support is a (preferably transparent) plastic polymer like polycarbonate, polyethylene or PPMA polymer containing olefinic groups or a solid support upon which olefinic groups are incorporated by chemical (grafting) reaction or by a physical deposition of a layer or dendritic compounds bearing olefinic molecules, for instance by the addition of a chlorosilane derivative.

Please replace the paragraph beginning at page 5, line 18, with the following rewritten paragraph:

A17
Therefore, said capture molecules present upon the microarrays are specific for said complementary target molecules and are preferably parts of coupling pairs, such as complementary strands of nucleotide sequences, antibodies or active hypervariable portions of an antibody/antigenic structure or haptens, receptors/ligands, biotin/streptavidin molecules, possibly coupled with other chemical or biochemical molecules or any double pairs binding system suitable for the identification, characterization, screening and recovery of biological or chemical libraries of molecules, for biomedical analysis such as gene expression analysis, polymorphism or mutation detection, molecular diagnostic, DNA sequencing and gene characterization.

Please replace the paragraph beginning at page 6, line 4, with the following rewritten paragraph:

A18
Brief Description of the Drawings

Please replace the paragraph beginning at page 6, line 5, with the following rewritten paragraph:

A19
Fig. 1 gives a schematic presentation of the functionalization reaction for glass surface.

Please replace the paragraph beginning at page 6, line 7, with the following rewritten paragraph:

A20
Fig. 2 demonstrates the fixation capacity of the functionalized glass for aminated DNA nucleotide sequences.

Please replace the paragraph beginning at page 6, line 9, with the following rewritten paragraph:

A21
Fig. 3 demonstrates the fixation yield of antibodies captured after spotting on functionalized glass which have reacted with protein A.

Please replace the paragraph beginning at page 6, line 12, with the following rewritten paragraph:

A22 Detailed Description of the Preferred Embodiments

Please replace the paragraph beginning at page 6, line 18, with the following rewritten paragraph:

According to the invention, the olefinic groups present on the surface of said solid support are oxidized into aldehyde in the presence of low concentrations of permanganate and periodate in a buffered aqueous solution, which can be advantageously applied to most of the plastic polymers without damaging the chemical or even more the physical properties of the polymer.

Please replace the paragraph beginning at page 6, line 23, with the following rewritten paragraph:

Other transparent polymers like PMMA or polyethylene are also well suited for functionalization according the process of the present invention.

Please replace the paragraph beginning at page 6, line 29, with the following rewritten paragraph:

In one embodiment of the invention, glass is used as support for biochip microarray construction by first attachment of olefinic groups using chlorosilane derivatives and then oxidation of these olefinic groups into aldehyde (Fig. 1). Preferably, the olefinic groups are distant from the solid support by a spacer of at least 2 atoms. Example 4 shows that olefinic groups present at the extremity of a chain of 4 or 6 carbon atoms give much better hybridization yield than olefinic groups with only one carbon as spacer from the hydroxyl of the glass.

Please replace the paragraph beginning at page 7, line 3, with the following rewritten paragraph:

In another embodiment of the invention, acrylic-polyacrylic resins are oxidized by the same method. These acrylic-polyacrylic resins have been successfully oxidized when present on another support like polycarbonate of the CDs. The fact that no organic solvent is used, makes the method well adapted for supports like polycarbonate. The present invention is particularly well suited for the oxidation of olefinic groups present at the extremities of long or dendritic molecules, since the presence of a spacer from the surface was found to be very favorable for the DNA hybridization yield (see also example 4).

Please replace the paragraph beginning at page 7, line 11, with the following rewritten paragraph:

A27

Mild oxidation of olefinic groups into aldehydes can also be performed with oxidative methods such as ozonolysis. The results obtained were however less quantitative. A decrease of about 20 %. The transfer of such method to industrial production is however much more complicated to implement with the ozonolysis method than with the permanganate/periodate. The main reasons are the fact that the oxidation has to be performed in organic solvent and a low, well controlled temperature. was observed with ozonolysis treatment than with the permanganate/periodate oxidation. In a preferred embodiment of the invention an aldehyde bearing surface can be used for the deposit of DNA aminated capture nucleotide sequences. The reaction of the amino groups with the aldehyde is a fast reaction making the invention well suited for microarray construction by using small solutions performed at room temperature and submitted to evaporation. Microarray construction with spots of between 0.05 to 0.5 mm used droplet or pin deposit droplet in the range of 0.1 to 5 nl. In another embodiment, the imine formed bond is then reduced by incubation with sodium borohydride or another reducing agent in order to stabilize the bond and inactivate the excess of free aldehyde.

PLEASE REPLACE THE PARAGRAPH BEGINNING AT PAGE 7, LINE 27, WITH THE FOLLOWING REWRITTEN PARAGRAPH:

A28

In another embodiment of the invention, molecules are bound to the aldehyde surface, being the first member of a binding pair. The second member being the molecule to be detected or identified or quantified in biological or chemical samples.

PLEASE REPLACE THE PARAGRAPH BEGINNING AT PAGE 7, LINE 30, WITH THE FOLLOWING REWRITTEN PARAGRAPH:

A29

Preferably, the first member is an antigen (hapten) or antibody, a ligand or a receptor, a biotin or a streptavidin but also peptides, proteins or DNA which are recognized by complementary or other binding molecules. For example DNA specific sequences attached to a support can be used to detect DNA binding proteins. One specific application is the detection of transcriptional factors.

PLEASE REPLACE THE PARAGRAPH BEGINNING AT PAGE 8, LINE 3, WITH THE FOLLOWING REWRITTEN PARAGRAPH:

A30

The invention is particularly well suited for construction of large numbers of bound molecules on the same surface and its automation. Thus libraries of chemicals, peptides, ligands, antigens are easily constructed on such support given the facility of deposit of molecules by

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robot. The solid supports are then easily used for screening libraries of molecules either biologically (like clones, plasmids bank or phage display molecules) or chemically constructed. Chemical libraries are now easily constructed due to the progress in the combinatory or parallel synthesis of molecules.

Please replace the paragraph beginning at page 8, line 13, with the following rewritten paragraph:

A31
Ethanol, Maleic acid, NaCl, and SDS (sodium dodecyl sulfate), are from Merck (Darmstadt, Germany). NaBH₄, Tween 20, streptavidin-cy3 and streptavidin-gold are from Sigma (St Louis, MO, USA). [α -³²P] dCTP are from Dupont de Nemours (Boston, MA, USA). Oligonucleotides are from Eurogentec (Seraing, Belgium). Hybridization chambers of 65 μ l are from MJ Research INC (Watertown, Ma, USA). Oligo dT nucleotide sequence, the reverse transcriptase Superscrip II and Rnase H are from Gibco BRL (Paisley, UK)). Rnasin ribonuclease inhibitor is from Promega (Madisson, USA). Silylated (aldehyde) slides are from Cell Associates (Houston, TX, USA).

Please replace the paragraph beginning at page 8, line 22, with the following rewritten paragraph:

A32
The arrayer and colorimetric microarray reader are from WOW (Naninne, Belgium) using 250 μ m pins from Genetix (UK). The liquid scintillation analyzer LS 60001C is from Beckman Instruments (Fullerton, CA, USA); Aqualuma is from Lumac LSC (Groningen, Netherlands). High Pure PCR Product Purification Kit, dNTP, uracil-DNA-Glycosylase and Biotin-16-dUTP are from Boehringer (Mannheim, Germany). Hybridization solution and silver blue revelation solution are from AAT (Namur, Belgium). Taq DNA polymerase is from Biotools (Spain). 9600 thermocycler is from Perkin Elmer (Foster City, CA, USA). The microarray fluorescent reader is a array-scanner GSM 418 from Genetic Microsystem (Woburn, MA, USA). Allyltrichlorosilane, 5-hexenyltrichlorosilane and 7-octenyltrichlorosilane are from ABCR (Germany, Karlsruhe). Potassium permanganate, sodium periodate and toluene are from Aldrich chemical (Milwouka, Wi, USA). Non treated glass slides were purchased from Knittel glaser (Germany).

Please replace the paragraph beginning at page 9, line 11, with the following rewritten paragraph:

A33
The olefinic functions present either on glass or polymers were oxidized in the following way. The slides were dipped into a solution of 0.1M Phosphate buffer at pH 7.5 containing 20

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mM NaIO₄ and 0.5 mM KMnO₄ under mild agitation during 1h, washed twice with water, dried under Nitrogen flow and stocked under vacuum. Glass slides activated in this way were called diaglass slides.

Please replace the paragraph beginning at page 9, line 25, with the following rewritten paragraph:

A34
Radioactive labeling is carried out by the incorporation of [α -³²P] dCTP during the PCR amplification. Amplified DNA are separated from unincorporated nucleotides and primers by chromatography on High Pure PCR Product Purification Kit. DNA concentration is then measured by its absorbency at 260 nm. The purity of the fragment is checked by agarose gel electrophoresis.

Please replace the paragraph beginning at page 10, line 19, with the following rewritten paragraph:

A35
The first step of the functionalization is the grafting on the glass slides of three ω -olefinic silane coupling agents having different chain length. These were allyltrichlorosilane (C3), 5-hexenyltrichlorosilane (C6) and 7-octenyltrichlorosilane (C8).

Please replace the paragraph beginning at page 10, line 25, with the following rewritten paragraph:

A36
The protocols for capture nucleotide sequence synthesis and fixation on glass slides are described in example 3. The only difference is that the capture nucleotide sequence is multibiotinylated and not radiolabeled and were spotted at 200 nM.

Please replace the paragraph beginning at page 10, line 28, with the following rewritten paragraph:

A37
Slides are incubated 45 min at room temperature with 800 μ l of streptavidin-cy5 conjugate. After incubation, slides are washed 5 times 1 min with buffer 1, then rinsed twice with water. The detection is performed using the array-scanner GSM 418. Each spot was then quantified by a home made quantification software. The results gave a value of fluorescence of 2 for the C3, 234 for the C6 and 242 for the C8 ω -olefinic silane coupling agent.

Please replace the paragraph beginning at page 11, line 7, with the following rewritten paragraph:

A38
Cytomegalovirus DNA sequence is used as template for target DNA production. Targets are synthesized by PCR using primers and method described elsewhere (Zammatteo, et al..

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(1997). *Anal. Biochem.* 253, 180-189). Target are 437 pb in length. Labeling is obtained by the incorporation of Biotin-16-dUTP in a ratio to dTTP of 1:1 during the PCR amplification. DNA concentration is then measured by its absorbency at 260 nm. The purity of the fragment is checked by agarose gel electrophoresis.

Please replace the paragraph beginning at page 11, line 13, with the following rewritten paragraph:

A39

The hybridization solution is composed of 2XSSC pH 7, 4% SDS, 100 µg/ml salmon sperm DNA and 10 nM of 437 pb biotinylated targets CMV in a final volume of 70 µl. This solution is loaded on the array framed by an hybridization chamber which is then sealed by a coverslip. Slides are then placed on a heating block for 5 min at 98°C to denature target amplicons. Hybridization is carried out at 50°C for 2 h. Slides are then washed 4 times and the fluorescence measured as in example 4. The results gave a value of 217 for the hybridization performed on the Diaglass slides and 46 for the Telechem.

Please replace the paragraph beginning at page 11, line 24, with the following rewritten paragraph:

A40

Reverse transcription is done on 2 µg of mRNA extracted from hepatocytes in primary culture using the following procedure.

Please replace the paragraph beginning at page 11, line 26, with the following rewritten paragraph:

A41

In a sterile, nuclease free microtube, 1 µg of the oligo dT nucleotide sequence is added to mRNA extracted from rat liver. Nuclease free water is added to obtain a final volume of 10µl. This mixture is denatured for 10 min at 70°C and then chill on ice for 5 min. The reverse transcription is performed by adding the following components to the annealed nucleotide sequence template : 4 µl of First Strand Buffer supplied with the reverse transcriptase (250 mM Tris-HCl pH8,3, 375 mM KCl, 15 mM MgCl₂), 2µl of DTT 0,1M , 40 units of Rnasin ribonuclease inhibitor, 500 µM dATP, 500 µM dCTP, 500 µM dGTP, 130 µM dTTP, 70 µM biotin dUTP. The reaction mixture is gently mixed by flicking the tube and incubated for 2 min at 42°C. 300 units of reverse transcriptase SuperScript II are added to the mixture and tubes are incubated at 42°C for one hour. The reaction is stopped by heating at 70°C for 15 min. To remove RNA complementary to the cDNA, a treatment with Rnase H is performed at 37°C for 20 min.

Please replace the paragraph beginning at page 12, line 9, with the following rewritten paragraph:

A42
The hybridization protocol is described in example 5 using as target the total reverse transcription product and 2nM biotinylated CMV amplicons as positive control. Capture nucleotide sequence corresponding to the positive control is included on the array. The hybridization is carried out for 16 h at 60° C. The results were obtained in fluorescent scanning and are presented in figure 2. The maximum hybridization was obtained using 6 times less capture nucleotide sequence concentration for the Diaglass than for the Telechem.

Please replace the paragraph beginning at page 12, line 19, with the following rewritten paragraph:

A43
Capture nucleotide sequence immobilization

Please replace the paragraph beginning at page 12, line 23, with the following rewritten paragraph:

A44
The target DNA is a fragment (587 bp) of the femA gene sequence from *S. aureus* which is obtained by PCR using the following degenerated primers:

Please replace the paragraph beginning at page 13, line 4, with the following rewritten paragraph:

A45
The hybridization protocol was described in example 5. Slides are incubated 45 min at room temperature with 800 µl of streptavidin labeled with colloidal gold 1000X diluted in buffer 2. After incubation, slides are washed 5 times 1 min with buffer 1, then rinsed once with water. Gold catalyses silver precipitation using silver blue revelation solution (AAT,; Namur, Belgium). The slides are incubated 3 times 10 min with 800 µl of revelation mixture, then rinsed with water, dried and analyzed using a microarray reader. Each spot are then quantified by a home made quantification software. The results gave a hybridization value of 181 for the Diaglass slides and 111 for the Telechem.

Please replace the paragraph beginning at page 13, line 19, with the following rewritten paragraph:

A46
The hybridization protocol is described in example 5. 30 nM of biotinylated target DNA of 27 bases are hybridized on the array for 30 min at 50° C and the detection was performed as in example 7. The results gave a hybridization value of 238 for the Diaglass slides and 61 for the Telechem.

Please replace the paragraph beginning at page 13, line 25, with the following rewritten paragraph:

A47
The presence of proteinA fix antibody by their Fc fragments which facilitate antibody/antigen recognition antigen. Covalent fixation occurs between free amines of proteinA and aldehyde functions of glass slides.

Please replace the paragraph beginning at page 14, line 11, with the following rewritten paragraph:

A48
The different steps of the functionalization of the glass surface were characterized by X-ray photoelectron spectroscopy (XPS) and contact angle measurements. The C 1s line for Diaglass and Telechem slides can be resolved in two components, one at 285.0 eV corresponding to carbon in an aliphatic environment (CH_x) and an other, characteristic of the carbonyl from the aldehyde function at 288.7 eV. Furthermore, Diaglass slides reacted positively to the Tollens test (characteristic test of aliphatic aldehydes), giving a silver mirror. On the other hand, glass slides alone or with carboxylic acid or alcohol functionalization do not give positive result in this test.

Please replace the paragraph beginning at page 14, line 27, with the following rewritten paragraph:

A49
The level of aldehyde on the surface influence the yield of capture probes which are bound on the chips. This has been illustrated in example 3. This yield of capture DNA probe fixation in spotting conditions is a good characterization method of the aldehyde content of the glass since it gives the exact estimation of the final result expected for the use of these glasses. A yield of 67 % fixation was currently obtained in these working conditions with spotting of 150nM capture probes solutions versus 10 % for the telechem glass slides.

Please replace the paragraph beginning at page 17, line 3, with the following rewritten paragraph:

A50
**METHOD FOR OBTAINING A SURFACE ACTIVATION OF A SOLID
SUPPORT FOR BUILDING BIOCHIP MICROARRAYS**

Please replace the paragraph beginning at page 17, line 6, with the following rewritten paragraph:

A51
The present invention is related to a method for making microarrays comprising the steps of :